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COMPLEMENT SYSTEM DEFICIENCY ASSAY

The invention refers to assaying the complement system. More specifically, the invention refers to a method of functionally determining deficiencies in the complement system by only assaying the classical pathway, the alternative pathway, or the lectin pathway, respectively.

The complement system is a complex part of the innate immune system, which comprises a number of plasma proteins and transmembrane proteins that interact with each other. The term innate immunity is used in order to differentiate this type of immunity from that termed adaptive immunity. However, complement also plays a role in adaptive immunity, but is much less understood in this connection.

A series of soluble proteins present in serum aid, when activated, in the elimination of microorganisms and other antigens from tissues and from the blood. This is achieved either by the complement components alone, or by their subsequent interaction with cells expressing complement receptors which triggers other arms of the immune response.

In order to prevent host tissues from being damaged, the complement system has to be strictly regulated. The large number of proteins, present either in serum or expressed on cell surfaces are involved as complement regulatory molecules in the protection of host cells, in the control of complement during activation by antigen, and in the de-activation of complement once antigen has been eliminated.

As a major component of host defence against pathogenic organisms, the activation of the complement system is of key importance for the innate defense against invading pathogens. In higher organisms, animals respond to the antigenic challenge presented by invading organisms by the development of a specific immune response. Antibody is formed and cells that have the capacity to recognize the

specific foreign antigen are generated. Thus, the complement identifies its target *via* direct recognition of the microbial surface by complement components, as well as *via* indirect binding to adaptor molecules such as antibodies or acute phase proteins. Subsequent activation of the complement system generally results into elimination of the activator *via* humoral and cellular mechanisms. These defense functions of the complement system are required for optimal host immunity.

For example, the mannan-binding lectin (MBL) is able to bind to repetitive saccharides frequently present on the surface of many clinically relevant microorganisms. This direct binding of MBL is involved in the elimination of pathogens by the immune system. The importance of MBL in innate resistance against invading pathogens is clearly illustrated by persons with genetic mutations in the MBL gene. These mutations lead to structural abnormalities of the MBL molecule, resulting in impaired complement activation *via* the lectin pathway, which is associated with an increased susceptibility to infections.

The activation of the complement system is an important component of host defense. Following infection, triggering of the complement activation cascade *via* direct binding of complement components to a microbial surface may lead to opsonisation and pathogen elimination *via* humoral and cellular mechanisms. Furthermore, complement activation may trigger and amplify the acquired immune system.

In addition to playing an important role in host defense against infection, the complement system is a mediator in both the pathogenesis and prevention of immune complex diseases. It has a protective effect when functioning in moderation against appropriate pathogens, since an inflammation promoted by complement activation at the same time can result in cellular damage when not properly controlled.

The cascade reaction of complement activation can be triggered *via* at least three known activation pathways, i.e., the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP). These three pathways converge at the component C3. The terminal complement pathway consists of all proteins activated after C3, and results into assembly of the C5-9 group of proteins into the membrane attack complex (MAC). The MAC exerts powerful killing activity by creating perforations in cellular membranes.

Defects in the complement system may lead to a partial or complete blockade of the complement activation cascade. Depending on the level of the defect, either the induction phase or the effector phase of complement activation may be hampered, and the defect may affect more than one pathway. An impaired function of the complement system may occur due to genetic defects, or due to acquired deficiencies of complement components. Acquired complement deficiencies may occur due to formation of autoantibodies to complement components or due to excessive complement consumption. Genetic complement deficiencies have been described at all levels of the system.

Most complement defects are associated with disease, ranging from a relatively mild increase in the susceptibility to infections to the occurrence of a severe systemic autoimmune syndrome. Furthermore, an impaired complement function is associated with the occurrence of flares in patients with systemic lupus erythematosus (SLE). Therefore, functional assays to measure complement activity in human serum have a clear diagnostic and prognostic value.

However, during the last years it has become increasingly clear that complement components *via* similar mechanisms may target its effects to damaged self tissue. Thereby, the complement contributes to the amplification of tissue damage and inflammation in conditions such as

autoimmune diseases, immune complex diseases, Alzheimer's disease, and ischemia/reperfusion injury as occurring in e.g. myocardial infarction, stroke, and major surgery. For example, recent studies have also provided evidence that activation of the lectin pathway by MBL can be responsible for complement activation and related inflammation in ischemia/reperfusion injury as well as myocardial infarction. Furthermore, complement activation contributes to the pathogenesis of allograft and xenograft rejection. Thus, undesired activation of complement is involved in inflammation and associated tissue damage in a number of pathological conditions.

It is difficult to pinpoint and functionally determine deficiencies in the complement system. Defects may lead to a block in the complement cascade at the point of the defect. In general, patients with defects in the classic pathway proceed down the pathway until the point of defect, and the latter proteins in the cascade are not recruited. On the other hand, individuals with abnormalities of the alternative pathway are less common than are individuals with abnormalities of the classical pathway and, in some cases, only a few individuals with the abnormality have been described.

Furthermore, deficiencies in the complement cascade can lead to overwhelming infection and sepsis. Deficiencies in complement mainly predispose patients to infection *via* two mechanisms, i.e. ineffective opsonization and defects in lytic activity (defects in MAC).

An example is defects that result in inadequate opsonization. Opsonization is the process of coating a pathogenic organism so that it is more easily ingested by the macrophage system. The complement protein C3b, along with its cleavage product C3bi, is a potent agent of opsonization in the complement cascade. Any defect that causes decreased production of C3b results in inadequate

opsonization ability. Such opsonization defects can be caused by deficiencies in components of the classical, alternative, or MBL pathways, or defects may be caused by deficiencies of the C3b component itself.

The complement function is mostly measured by using hemolytic assays, which enable the functional assessment of the classical complement pathway and the alternative complement pathway, respectively. In these hemolytic assays, the function of the complement pathways is expressed as its ability to generate the C5b-9 complex upon activation. Such an assay is currently not available for the lectin pathway of complement.

Usually, a method for detecting complement or its deficiency in the blood is performed by means of tedious haemolytic and antigenic methods. Increased or decreased levels of different components of complement pathways are assayed. Such tests require targeting antibodies, which will recognize specific complement proteins. Generally, antigenic assays of complement proteins in serum or plasma are the most readily available tests, particularly for C3. In the latter assays, the serum level of C3 is provided, but it tells little or nothing about the functional activity. Hypofunctional variants exist, but no non-functioning C3 variants are described.

When studying functional complement, sheep erythrocytes have been used, since they are easily lysed by antibody and complement. The most commonly performed test for functional complement activity is the CH_{50}, a measure of the ability of a dilution of the serum of a patient in order to lyse an antibody coated sheep erythrocyte. When for example one of the proteins of the classic pathway is missing, lysis in the CH_{50} assay is blocked, the functional titer of the deficient protein being close to zero, and the CH_{50} obtained is zero. An alternative pathway lytic test

exists and is termed the AP_{50}. This test is less sensitive than the CH_{50} test and is used as a screening test.

Thus, there is a need of methods for the functional identification of deficiencies in the complement system of a mammal, including humans.

The purpose of the invention is to achieve a method of functionally determining deficiencies in the complement system whereby the above-mentioned problems are eliminated.

In order to achieve this purpose, the method according to the invention has been given the characterizing features of claim 1.

According to the invention a method for functionally determining deficiencies in the complement system is provided by only assaying the classical pathway, the alternative pathway, or the lectin pathway, respectively. In the inventive method a sample of mammalian blood, serum, plasma, or another body fluid is first provided, and one of the classical pathway (CP), the alternative pathway (AP), or the lectin pathway (LP), to be functionally assessed, is selected. The activation of the two non-assayed pathways is prevented in the sample, and the pathway to be assayed is activated. At last, any activation of the complement pathway in the sample is determined.

In the inventive method potent and specific complement inhibitors are used to prevent undesired activation of each complement pathway.

In the classical complement pathway the recognition unit is C1q. C1q is strongly related to the family of proteins known as collectins, which have a complex structure made up of trimers consisting of, a segment of a collagenous sequence at its N-terminus, and a C-type lectin domain at its C-terminus. C1q does not possess a lectin domain, but shares many structural and functional features with the collectins. The plasma concentration of C1q amounts to around 100 µg/ml, and *in vitro* experiments

show that only a small fraction of C1q is sufficient for a complete complement activation.

At least two different types of inhibitors of C1q can be used to prevent the activation of the classical pathway, those binding to the globular heads and interfering with ligand recognition, and those binding to the collagenous tail and impairing the interaction with complement activating enzymes and/or C1q receptors. Obviously, those inhibitors which interfere with ligand binding inhibit an earlier step of classical pathway activation. On the other hand, molecules that bind to the globular head of C1q may trigger a C1 activation in the fluid phase, especially when these C1q-binding molecules are multimeric.

Preferably, monoclonal antibodies directed against C1q are used in order to efficiently inhibit C1q-mediated ligand binding and complement activation.

A number of identified molecules are shown below in Table 1, which can regulate the functional activity of C1q.

Table 1.

Inhibitor	**Description/ comments**	**Mechanism of C1 inhibition**
C1 inhibitor	Plasma serine protease inhibitor	Inhibits C1r and C1s activity
IVIg	Has broad activity	Blocks C1q ligand binding
CRT	Contains several active domains	May inhibit both C1q head and C1q tail
C1Qr	Native C1q receptor	Binds C1q tail, inhibits C1 formation
E. coli C1q binding protein		Binds C1q tail, inhibits C1 formation
gC1qR	Native C1q receptor	Binds C1q head
Decorin	Matrix protein	Binds to C1q head and tail preparations
Chondroitin sulphate proteoglycan	plasma proteoglycan/B cell-secreted	Inhibits C1 formation

Table 1 (cont.)

Inhibitor	Description/ comments	Mechanism of C1 inhibition
Surfactant protein A	Collectin present in the lung	Inhibits C1q ligand binding and C1 formation
HNP-1	Cytotoxic peptide produced by neutrophils	Binds C1q tail and inhibits C1 formation
Peptide gC1q-R_{18} (TDGDKAFVDFLSDEIKEE)	Derived from gC1qR	Not defined
Peptide KDIRCKDD	Derived from CRT	Inhibits C1q ligand binding
Peptide AEAKAKA	Derived from human IgG	Inhibits C1q ligand binding
Peptide VQVHNAKTKPR	Derived from human IgG1	Not defined
Peptide WY	Derived from human IgG	Inhibits C1q ligand binding
Peptide 2J (CEGPFGPRHDLTFCW)	Synthetic peptide	Binds C1q head, inhibits ligand binding
ghB3	Trimeric C1q B chain	Acts as a competitor for C1q binding
Peptide CBP2 LEQGENVFLQATLL	Derived from C1q B chain	Acts as a competitor for C1q binding

In Table 1, natural C1q-binding molecules, several series of C1q-binding peptides, and competitive inhibitors derived from the sequence of C1q are shown, which can be used in the method according to the invention for inhibiting C1q of the complement system or as a inhibitory C1q-binding protein when preventing the activation of the classical pathway.

The classical C1q-binding proteins are immunoglobulins, and the globular head domain of C1q interacts with both IgG and IgM upon antigen binding, or after its aggregation or immobilization. Human immunoglobulin for intravenous use (IVIg) can inhibit complement activation, and the main mechanism of action seems to be a scavenging of C1q and activated C4 and C3 by soluble immunoglobulins.

Next to immunoglobulins, a number of other proteins have been identified that are able to bind C1q. Among these are C1q-binding proteins, which (under certain conditions and on certain cell types) are present on the cell membrane, such as calreticulin (CRT), the endothelial C1q receptor, and the globular C1q receptor (gC1q-R). The membrane-expressed forms of these C1q binding proteins are involved in C1q-mediated cell activation, whereas soluble forms of these molecules are able to inhibit C1q function.

Calreticulin (CRT) is a calcium-binding protein that is mainly present in the lumen of the endoplasmic reticulum. Protein sequencing data indicate that CRT probably is identical to the C1q receptor present on the cell surface of various cell types. CRT can bind to the α2 macroglobulin receptor (CD91) at the cell surface, and different domains of CRT can be distinguished, which bind to C1q, i.e. the adjacent N domain and P domain, but not the C domain. Furthermore, the S-domain, which overlaps parts of the N- and P-domains, also shows clear C1q binding. The S-domain of CRT clearly resembles a CUB domain present in C1r and C1s, suggesting that this domain may interact with the collagenous part of C1q.

Accordingly, different sites on C1q interact with different domains of CRT. Native and recombinant CRT, as well as the N-domain, the P-domain and the S-domain, all inhibit the C1q-dependent hemolysis as well as the formation of C1. A number of C1q-binding peptides have also been identified that are able to inhibit C1q function, which peptides are useful in the present inventive method. Among these are human neutrophil peptide-1, peptides derived from natural C1q-binding proteins, and synthetic peptides selected from peptide libraries.

A C1q-binding protein (gC1qR) can also be used, which binds specifically to the globular head of C1q. The native

C1q receptor (C1qR), isolated from human endothelial cell membranes or from polymorphonuclear leukocyte membranes, functionally inhibits the formation of active C1. This inhibitory activity is reversed by C1q collagenous tails, but not by globular heads. In a similar way, a soluble protein isolated from E. coli, which binds C1q, is able to inhibit C1 formation.

In addition, the activation of the classical pathway can be prevented by providing in the sample an antibody directed against C1r or C1s. In this connection several other C1q-binding molecules can be used, which can modulate C1q function. Examples are the C1q-associated plasma proteoglycan and the chondroitin sulfate proteoglycan produced by human B cells, which can bind C1q and inhibit C1 formation. The dermatan sulfate proteoglycan decorin, a constituent of the extracellular matrix, as well as the related proteoglycan biglycan are also suitable inhibitors.

Likewise, the activation of the classical pathway can be prevented by providing in the sample a peptide inhibitor of C1r or C1s. Several members of the pentraxin family, i.e. C reactive protein, serum amyloid P component and pentraxin-3, have been described to bind C1q. Pentraxin-3 can inhibit C1q activity under certain conditions, and surfactant protein A, a member of the collectin family, is able to bind to C1q and to inhibit its activity. This is accomplished by interfering in both the binding of C1r and C1s as well as the binding of immune complexes.

A competitive inhibition of C1q by C1q-derived molecules is an alternative approach for the inhibition of the classical complement pathway. Here, functionally inactive parts of the C1q molecule are used, which each serves as a competitive inhibitor for C1q ligand binding. Recombinant globular head domains of the C1q A (ghA) and B chain (ghB) have been generated, which separate domains are both able to bind to IgG, but the B domain is more potent

than the A domain. A better activity is obtained when the recombinant C1q B chain is trimerized by using the neck region of the surfactant protein D.

A small inhibitory C1q-binding molecule can also be used, such as the human neutrophil peptide-1 (HNP-1), which can bind to C1q and inhibit the classical complement pathway. This peptide belongs to the α defensin family of small cationic peptides, which are present in azurophilic neutrophil granules. It is preferred that such a peptide inhibitor of C1r or C1s is a synthetically produced peptide in order to achieve sufficient amounts at low costs.

Several C1q-binding peptides have been identified on basis of the amino acid sequence of C1q binding proteins. By using 92 overlapping peptides, several C1q binding sites in the N and P regions of CRT have been identified. A number of these peptides are able to inhibit a classical pathway activation in human serum as well as the binding of C1q to IgG. These peptides are characterized by a motif that resembles a binding site for C1q in the CH2 domain of IgG (ExKxKx).

In this connection, peptides directly derived from IgG have been described to inhibit C1q, such as a 7-meric peptide (i.e. AEAKAKA) containing the ExKxKx motif, an 11-meric peptide (VQVHNAKTKPR) derived from IgG1 that is related to the same motif, and a dimeric peptide (WY, c.f Table 1). These peptides were able to inhibit activation of the classical complement pathway in several *in vitro* assays. However, the WY peptide also inhibits the alternative complement pathway.

Among 42 peptides selected from phage-displayed peptide libraries based on phage binding to human C1q, 20 peptides have been identified, which can inhibit the classical complement pathway in human serum. Remarkably, 13 out of these 20 peptides were able to inhibit the classical pathway as well as the alternative pathway in hemolytic

assays, whereas 7 peptides specifically inhibited the classical pathway. Out of these peptides, the peptide 2J (CEGPFGPRHDLTFCW) was selected. Peptide 2J is a strong inhibitor of C1q hemolytic function. Similar to the peptides with an IgG motif, peptide 2J binds to the globular head of C1q and inhibits the binding of C1q to IgG. In addition, peptide 2J inhibits C1q from human, primate and rodent origin.

Other selected peptides useful for inhibiting the classical pathway are CEGPFGPRHDLTFCW, CRWDGSWGEVRC, CMWVRMWGDVNC, CFWAGKFGLGTC, CKDRWVVEERCC, and CWNRFKKMDRC. Several other peptides can also be used, which act as a competitor for C1q binding and are derived from the C1q B chain, e.g. the peptide CBP2 (LEQGENVFLQATLL).

The C1 inhibitor protein is a key molecule with respect to complement regulation at the recognition phase and inhibits the serine proteases of the activated C1 complex. Thus, any potentiator of the C1 inhibitor can be provided in the sample of the inventive method in order to prevent the activation of the classical pathway.

In addition, a protease inhibitor of C1r or C1s can be used, for example known inhibitors of serine proteases. These inhibitors can also be used when the activation of the lectin pathway is to be prevented.

If both these pathways are to be prevented, a Ca^{2+} binding agent can be used. Preferably, the binding agent is a chelating agent. Suitable chelating agents are amino-carboxylic acids, such as N-hydroxyethyliminodiacetic acid, nitrilotriacetic acid (NTA), ethylenediaminetetracetic acid (EDTA), N-hydroxyethyl-ethylenediaminetetraacetic acid (HEDTA) and diethylentriaminepentatetraacetic acid (DTPA).

A simple and effective way to prevent the activation of the alternative pathway is to dilute the sample. By addition of 1 M NaCl to a serum dilution buffer, C1q

binding and CP activation can be completely prevented whereas MBL binding can proceed.

In the alternative pathway, the serine protease factor D produces a C3 convertase which - if not inactivated - will continue to act on component C3 and cause its total depletion. Thus, the activation of the alternative pathway can prevented by providing in the sample a protease inhibitor of factor D of the complement system or an antibody directed against the same.

A specific inhibition of the lectin pathway of complement activation can be achieved by blocking the lectin domain of mannan-binding lectin (MBL) by carbohydrates , such as mannose, L-fucose, and N-acetylglucosamine. The mannan-binding lectin is a C-type lectin present in serum in a large pro-enzymatic complex that shows similarities with C1. Similar to C1q, MBL is a polymeric molecule of trimeric subunits. The trimers of MBL consist of three identical chains with a collagenous tail region and a carbohydrate recognition domain. In serum, MBL is associated with the MBL-associated serine proteases MASP-1, MASP-2, and MASP-3, which are responsible for the activation of C4, C2, and C3, respectively. These enzymes are homologous to C1r and C1s. MBL binding and complement activation can also be inhibited by using monoclonal antibodies directed against the lectin domain of MBL.

Thus, the activation of the lectin pathway is prevented by providing in the sample a carbohydrate or a derivative thereof. Preferably, the carbohydrate is a saccharide that is related to mannan. The carbohydrate derivative can also be a carbohydrate conjugate in order to increase the efficiacy when blocking the activation.

Likewise, the lectin pathway can be prevented by providing in the sample an antibody directed against MBL, an antibody directed against a MBL-associated serine

protease (MASP) protein, or a serine protease inhibitor of a MASP protein.

A peptide inhibitor of either MBL or MASP proteins can also be used for preventing the activation of the lectin pathway. For example, a peptide derived from human cytokeratin is able to inhibit MBL binding to its ligands as well as to inhibit endothelial complement binding *in vitro*. Similar data has been obtained with a plant lectin, *Ulex europaeus* agglutinin II, which is able to competitively inhibit MBL binding.

The classical pathway and the lectin pathway are differentially sensitive to the action of various natural and synthetic protease inhibitors. Thus, an alternative mechanism to inhibit activation of the lectin pathway of complement is to inhibit the enzymatic activity of different MASP's. Aprotinin, a biological protease inhibitor present in serum and tissue, is able to inhibit the activation of the lectin pathway but not of the classical pathway. Specific inhibitors of the lectin pathway can also be used, which target the MBL-associated proteases but not the C1-associated proteases.

The activation of the classical pathway starts with the binding of a foreign antigen by specific antibodies to form immune complexes, e.g. IgM. Thus, the classical pathway can be activated by providing in the sample at least one immunoglobulin. Preferably, the immunoglobulin is immunoglobulin IgG or IgM.

The large multimeric protein complex C1 is composed of the subunits C1q, C1r, and C1s. Each immunoglobulin Fc region has a single C1q-binding site, and each C1q must bind to 2 heavy chains to be activated (thus either 2 IgG which become cross-linked or 1 IgM).

Accordingly, the classical pathway can be activated by providing in the sample an antibody directed against

C1q. The activating substance can also be a natural ligand of C1q or a C1q-binding peptide.

A variety of activators of the alternative complement pathway have been described, such as certain particulate polysaccharides, for example, bacterial (lipopolysaccharides, LPS), yeast (zymosan), or plant (inulin) polysaccharides, fungi, bacteria, viruses, and certain mammalian cells.

The alternative pathway can thus be activated by providing in the sample a lipopolysaccharide or a derivative thereof. Other carbohydrates or derivatives thereof can also be used. Such a preferred carbohydrate is inulin.

The lectin pathway is activated by the binding of the members of collectin family of proteins to specific carbohydrate moieties on invading pathogens. These then directly activate the components of the classical pathway, avoiding the need for specific antibodies. One member of the collectin family is mannan-binding lectin (MBL), which is found in serum and binds to terminal mannose groups on bacteria.

Accordingly, the lectin pathway can be activated by providing in the sample a mannan or a MBL-binding carbohydrate. Preferably, the MBL-binding carbohydrate is mannose or fucose. The lectin pathway can also be triggered by binding of a synthetic carbohydrate conjugate of a microbial polysaccharide.

Since ficolins, GlcNAc-binding lectins in serum, are considered to be lectins with the ability of activating the lectin pathway, the lectin pathway can also be activated by providing in the sample a ficolin-binding carbohydrate.

However, certain pathogens have the ability to directly activate the classical pathway, without the need for specific antibody interactions. Activating molecules include yeast cell walls, bacterial lipopolysaccharide (LPS) and the capsids of several viruses. Likewise, aggreg-

ates of immunoglobulins, for example, IgA or IgE are known to activate the alternative complement pathway. Thus, when preventing one pathway, an activator should not be used in the inventive method, which itself activates this pathway.

In the method according to the invention any activation of the complement pathway in the sample can be determined by establishing an activation of a complement protein from C4 to C9 of the same. Preferably, SC5b-9, the terminal complement complex, is determined since it is the soluble form of the membrane attack complex (MAC, C5b-9) that is formed when complement activation occurs by either pathway. The determination can be accomplished by providing in the sample antibodies against the formed C5b-9 complex.

In a complement function assay a certain density of an activating substance is required. For example, an antibody must be bound, e.g. to an antigen or to a plastic material, in order to activate complement. Likewise, any activation of the complement should be performed by determining the binding of any complement protein from C4 to C9; or of any formed C5b-9 complex, to the activating surface.

Thus, it is preferred in the method according to the invention that the activating substance is immobilised on an inert carrier. An immobilisation on the surface of a carrier is accomplished by using conventional techniques. The activating substance can be immobilised by means of covalent coupling to the carrier as bead or matrice/gel.

Suitable carriers are synthetic polymeric carriers, such as polypropylene, polystyrene, substituted polystyrene, e.g. aminated or carboxylated polystyrene, polyacrylamides, polyamides, polyvinylchloride etc, glass, agarose, nitrocellulose etc. The carrier can for example be a well of an ELISA plate.

Examples.

The method of the present invention will now be further illustrated by but is by no means limited to the following examples.

Materials and Methods.

Human materials.

Human serum was obtained from 70 healthy adult volunteers and immediately frozen at -80 °C in aliquots. Outdated healthy donor plasma was obtained from the Blood-bank Leiden-Haaglanden, Leiden, the Netherlands. From a patient with Kahler's disease of the IgM type, plasma was obtained that became available after a plasmapheresis treatment.

Anti-C1q and anti-MBL antibodies.

Monoclonal antibodies against C1q were produced in mice as described before (Hoekzema R., *et al. Mol. Immunol.* 25, 485-494, 1988). The anti-C1q mAb 2204 (IgG1) is directed against the globular head domain of C1q and is able to inhibit the binding of C1q to IgG, as well as C1q-dependent hemolysis (Roos A., *et al., J. Immunol.* 167, 7052-7059, 2001). For the purification of mAb 2204, gamma globulins were precipitated from ascites by using 50 % $(NH_4)_2SO_4$. The precipitate was dialyzed against 10 mM Tris containing 2 mM EDTA (pH 7.8) and subjected to anion exchange chromatography by using DEAE-Sephacel (Pharmacia, Uppsala, Sweden). Proteins were eluted by using a salt gradient and the fractions that showed binding of mouse IgG to C1q-coated ELISA plates in the presence of 1 M NaCl were pooled, concentrated, dialyzed against PBS and stored at -80 °C.

Polyclonal anti-C1q antibodies were produced in rabbits. New Zealand White rabbits were immunized (weekly for four weeks) with 180 µg C1q dissolved in complete Freunds adjuvant, resulting in antisera with a positive

titer on C1q-coated ELISA plates beyond 1/25,000. IgG was precipitated from rabbit serum by using 40 % $(NH_4)_2SO_4$ and purified by using DEAE-Sephacel as described above.

Starting from purified rabbit IgG anti-C1q, Fab fragments were generated by using papain. Therefore, IgG was dialyzed against 10 mM phosphate buffer containing 10 mM L-cysteine and 2 mM EDTA (pH 7.0). Subsequently, mercuripapaine (from Sigma) was added (1 % w/w of the protein content) followed by incubation for 16 hours at 37 °C. After dialysis against PBS, the sample was applied to Sepharose-coupled protein G (from Pharmacia, Uppsala, Sweden), and the fall through fractions, containing Fab fragments, were pooled, concentrated, and used for experiments. Analysis by non-reducing SDS-PAGE showed a prominent band at approximately 45 kD.

A mouse mAb directed against the lectin domain of human MBL (mAb 3F8) was kindly provided by Dr. G.L. Stahl (Harvard Medical School, Boston, Massachusetts, USA) (Collard C.D., *et al.*, *Am. J. Pathol.* 156, 1549-1556, 2000).

Preparation of human C1q and C1q-depleted serum.

Human C1q was isolated from human donor plasma exactly as described previously and was stored at -80 °C ((Roos A., *et al.*, *J. Immunol.* 167, 7052-7059, 2001). Isolated C1q was able to completely restore the lysis of antibody-coated erythrocytes induced by C1q-depleted human serum.

For the preparation of C1q-depleted serum, undiluted normal human EDTA plasma (obtained from a donor with the MBL/AA genotype) was applied on column consisting of Biogel A5 (from Biorad) coupled to rabbit IgG anti-human C1q. The column was washed by using Veronal-buffered saline (VBS; 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10 mM EDTA. Fractions were tested in a C1q-dependent hemolytic assay in the absence or

presence of purified C1q. Fractions that showed complete erythrocyte lysis in the presence of C1q, but not in the absence of C1q, were pooled and concentrated until the original volume. After recalcification, C1q-depleted serum was stored at -80 °C.

Isolation of human IgM.

Plasma containing an IgM paraprotein was dialyzed against 10 mM sodium acetate containing 2 mM EDTA (pH 5.0). The precipitated proteins were recovered by centrifugation, dissolved in PBS, dialyzed against Tris/EDTA buffer (10 mM Tris, 2mM EDTA, pH 7.8 and conductivity 5.0 mS), and subjected to anion exchange chromatography by using DEAE-Sephacel. The IgM eluted by the salt gradient was pooled, dialyzed against 10 mM sodium acetate (6.0 mS, pH 7.0) and applied to a CM-C-50 Sephadex anion exchange column (from Pharmacia). Following elution with a salt gradient, fractions containing IgM were pooled, concentrated, and applied to a Superdex 300 gel filtration column. Peak fractions containing IgM and free of IgG were pooled, concentrated, and stored at -80 °C.

In the drawings

Figure 1 shows anti-mannan-antibodies in human serum; A-C: Different concentrations of human serum from three different healthy donors were incubated on plates coated with either mannan (closed symbols, solid lines) or BSA (open symbols, dashed lines). Binding of IgG (A), IgA (B) or IgM (C) was detected. As a positive control, plates were incubated with pooled immunoglobulin, as indicated; D: Anti-mannan antibodies of the three major Ig classes were quantified in healthy donor serum (N = 70). Solid lines indicate the median concentrations, dashed lines indicate the detection limits.

Figure 2 shows the role of C1q in activation of the CP and the LP;
A, B: Normal human serum or C1q-depleted serum (C1qD-NHS),

diluted in GVB++, was incubated on plates coated with IgM (A) and mannan (B), respectively, followed by detection of C4 binding;

C: NHS and C1q-depleted NHS (diluted 1/400) were incubated on plates coated with IgM or mannan in the presence or absence of purified C1q (0.5 µg/ml), as indicated.

D: NHS was incubated on IgM- or mannan-coated plates in the presence or absence of blocking mAb directed against MBL (mAb 3F8, 10 µg/ml) or C1q (mAb 2204, 20 µg/ml), or both (combination).

Figure 3 shows complement activation *via* the LP and the CP;

Complement activation was induced by incubation of different concentrations of NHS on plates coated with IgM for CP activation (A) or with mannan for LP activation (B), in the presence or absence of mAb 2204 (20 µg/ml). Activation and binding of complement was demonstrated by detection of C1q, C4, C3, and C5b-9 using specific mAb.

Figure 4 shows activation of the alternative pathway; NHS was incubated on plates coated with mannan, LPS, or BSA, in a calcium-free buffer (GVB/MgEGTA) to block activation of the CP and the LP. Binding of C3 (A) and C4 (B) was subsequently assessed.

EXAMPLE 1. Assessment of functional lectin pathway activity by ELISA.

Functional activity of the lectin pathway was assessed with ELISA by using immobilized mannan as a ligand. Mannan was obtained from Sigma from Saccharomyces cerevisiae; M7504), dissolved in PBS (10 mg/ml) and stored at -20 °C. Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with mannan (100 µg/ml) in coating buffer (100 mM $Na_2CO_3/NaHCO_3$, pH 9.6) for 16 hours at room temperature or for 2 hours at 37 °C. After each step, the plates were washed three times with PBS containing 0.05 % Tween 20.

Residual binding sites were blocked by incubation for one hour at 37 °C with PBS containing 1 % BSA. Serum samples were diluted in GVB++ (VBS containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05 % Tween-20, and 0.1 % gelatin; pH 7.5) in the presence of mAb 2204 (20 µg/ml) as an inhibitor of C1q, unless otherwise indicated. This mixture was pre-incubated for 15 minutes on ice, before addition to the plates. The plates were then sequentially incubated for 1 hour at 4 °C and for 1 hour at 37 °C, followed by washing. Complement binding was detected by using mouse mAb conjugated to digoxygenin (dig) by using digoxygenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of C1q, C4, C3, and C5b-9 was performed by using mAb 2214 (anti-human C1q), mAb C4-4a (anti-human C4d), RFK22 (anti-human C3), and AE11 (anti-C5b-9), kindly provided by Dr. T.E. Mollnes, Oslo, Norway), respectively. Binding of mAb was detected by using dig-conjugated sheep anti-mouse antibodies (Fab fragments) followed by HRP-conjugated sheep anti-dig antibodies (Fab fragments, both from Boehringer Mannheim). All detection antibodies were diluted in PBS containing 1 % BSA and 0.05 % Tween 20. Enzyme activity of HRP was detected following incubation at room temperature for 30-60 min with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (from Sigma; 2.5 mg/ml in 0.1 M citrate/Na_2HPO_4 buffer, pH 4.2) in the presence of 0.01 % H_2O_2. The OD at 415 nm was measured by using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).

Quantification of anti-mannan antibodies in human serum.

For the quantification of anti-mannan antibodies in human serum, ELISA plates were coated with mannan and blocked with 1 % BSA in PBS. Serum samples were diluted 1/100 for detection of IgG anti-mannan Ab, 1/10 for detec-

tion of IgA anti-mannan Ab, and 1/40 for detection of IgM anti-mannan Ab, respectively, unless otherwise indicated. For quantification, pooled human IgG (48 mg/ml IgG), pooled human IgA (41 mg/ml IgA), and pooled human IgM (35 mg/ml IgM) were used as a standard for detection of IgG, IgA and IgM anti-mannan antibodies, respectively (kindly provided by Biotest Pharma GmbH, Dreieich, Germany). The concentration of anti-mannan antibodies in these preparations was arbitrarily set at 1000 U/ml. All samples were diluted in PBS containing 0.05 % Tween 20 and 1 % BSA. Antibody binding was detected by using biotinylated HB43 (mouse mAb anti-human IgG), biotinylated HB57 (mouse mAb anti-human IgM) and dig-conjugated 4E8 (mouse mAb anti-human IgA), respectively, followed by either HRP-conjugated streptavidin or HRP-conjugated sheep anti-dig antibodies (both from Boehringer).

Mannan is a major ligand of MBL that can efficiently activate the LP of complement. However, human serum contains anti-carbohydrate antibodies, probably resulting f rom previous microbial contacts. Such anti-carbohydrate antibodies may bind to mannan and the resulting immune complex may contribute to complement activation by mannan *via* activation of the classical complement pathway (Petersen S.V., *et al.*, *J. Immunol. Methods* 257, 107-116, 2001). Mannan-binding antibodies are clearly detectable in human serum as assessed by ELISA (Fig. 1). Incubation of pooled human IgG (Fig. 1A), IgA (Fig. 1B) and IgM (Fig. 1C) on immobilized mannan resulted in a dose-dependent binding of IgG, IgA, and IgM, respectively, as detected by isotype-specific mAb. As a control, parallel incubations were performed on immobilized BSA, resulting in low or undetectable background binding of pooled Ig. Incubation of three sera from healthy donors on mannan-coated plates resulted in a strong dose-dependent IgG binding in all three sera. In donor 1, IgA and IgM anti-mannan Ab were undetectable,

serum from donor 2 contained IgG, IgA, and IgM anti-mannan antibodies, whereas in donor 3 some IgM binding was observed but no IgA binding (Fig. 1A-C). Binding of Ig was undetectable following incubation of serum on BSA-coated plates (Fig. 1A-C). A quantification of anti-mannan antibodies in sera from 70 healthy donors is presented in Fig. 1D. IgG and IgM anti-mannan Ab were present in nearly all donors, with a large interindividual variation, whereas IgA anti-mannan Ab were detected in 63 % of the donors. No significant correlation was observed between the three major isotypes of anti-mannan antibodies, or between anti-mannan antibodies and MBL concentrations (not shown).

EXAMPLE 2. Functional characterization of the lectin pathway in the presence of C1q-inhibitory Ab.

Both the LP and the CP are calcium-dependent and lead to activation of C4. A distinction between both pathways can be made by selection of a specific ligand that induces specific activation of either the LP or the CP. In view of the presence of anti-mannan Ab in human serum, mannan is likely to activate both the LP, *via* MBL, and the CP, *via* anti-mannan Ab. Therefore, a strategy was developed to inhibit activation of the CP in order to allow solely the activation of the LP by immobilized mannan, by using inhibitory anti-C1q antibodies.

Anti-C1q antibodies were tested for their ability to inhibit the CP of complement by using immobilized IgM as a specific activator of the CP. Incubation of 1 % normal human serum (NHS) on immobilized IgM induces deposition of C4, which could be dose-dependently inhibited by the anti-C1q mAb 2204, by rabbit IgG anti-C1q antibodies and by Fab fragments prepared from this rabbit anti-C1q antibody preparation. Complete inhibition was reached when the antibodies were applied at 5 µg/ml. In contrast, rabbit IgG prepared from non-immunized rabbits did not have an effect

on C4 activation *via* the CP. These antibodies were tested for their effect on complement activation induced by immobilized mannan. Incubation of NHS on mannan induced a dose-dependent deposition of C4 with a maximal activation at a serum concentration of 1 %. Addition of a fixed concentration of mAb 2204, Fab anti-C1q fragments, or normal rabbit IgG as a control had a slight inhibitory effect on C4 activation. In contrast, rabbit IgG anti-C1q Ab induced complete inhibition of C4 activation by mannan, most likely due to complement consumption *via* C1q-anti-C1q complexes. These data show that C1q-inhibitory antibodies can block CP activation completely whereas mannan-induced activation of the LP can proceed in a C1q-independent way.

To further examine the role of C1q in complement activation by mannan and by IgM, NHS was depleted from C1q. Depletion of C1q from NHS resulted in a complete inhibition of C4 activation by immobilized IgM (Fig. 2A), as previously described (Petersen S.V., *et al.*, *J. Immunol. Methods* 257, 107-116, 2001), whereas C4 activation by immobilized mannan was slightly inhibited by depletion of C1q (Fig. 2B). Reconstitution of C1q-depleted serum with purified C1q resulted in a complete restoration of C4 activation by IgM (Fig. 2C). In contrast, C4 activation by mannan was slightly inhibited by the addition of purified C1q to C1q-depleted serum, possibly due to the presence of an inhibitory protein co-isolated with C1q. The contribution of C1q and MBL to C4 activation by IgM and mannan was further studied by using blocking mAb against C1q and MBL, respectively (Fig. 2D). C4 activation on IgM-coated plates was completely inhibited by mAb anti-C1q and no inhibition occurred with a blocking anti-MBL mAb. In contrast, the C4 activation induced by mannan was partially inhibited by mAb anti-C1q and strongly inhibited by mAb anti-MBL. A complete inhibition of mannan-induced C4 activation was achieved when a combination of mAb anti-C1q and mAb anti-

MBL was used. Together, these data indicate that IgM-mediated activation of C4 is completely dependent on C1q and does not involve MBL. In contrast, mannan-induced activation of C4 is mainly mediated by the LP but comprises a minor contribution of the CP. The latter contribution of the CP can be inhibited by C1q-blocking Ab, thus allowing activation of the LP only.

EXAMPLE 3. Complement activation and formation of C5b-9 via the CP and via the LP.

The complement activation cascade was further studied by using mAb to detect binding of specific complement components upon their activation *via* the CP and the LP, respectively. Incubation of NHS on immobilized IgM resulted in a dose-dependent deposition of C1q, C4, C3, and C5b-9 to the plate (Fig. 3A). Binding of C1q and a subsequent complement activation induced by IgM could be completely inhibited by mAb 2204. Incubation of NHS on immobilized mannan resulted in a dose-dependent binding of C4, C3 and C5b-9, whereas binding of C1q was hardly detectable (Fig. 3B). Complement activation by mannan was only slightly inhibited by the addition of mAb 2204. Therefore, addition of mAb 2204 to serum allows the specific detection of LP activation by using mannan as a ligand without any interference of the CP.

EXAMPLE 4. Assessment of functional alternative pathway activity by ELISA.

The protocol for the functional activity of the alternative pathway was similar to the protocol for the LP assay, Example 3, with important modifications. As a ligand for AP activation, LPS was coated at 10 µg/ml. LPS from *Salmonella typhosa* was obtained from Sigma (L-6386), dissolved in PBS at 1.6 mg/ml and stored at -20 °C. Plates were blocked by using 1% BSA in PBS. Serum samples were diluted in GVB/MgEGTA (VBS containing 10 mM EGTA, 5 mM

$MgCl_2$, 0.05 % Tween-20, and 0.1 % gelatin; pH 7.5) and incubated in the plate for 1 hour at 37 °C. Complement binding was assessed by using dig-conjugated mAb directed against C4 and C3 followed by the detection of mAb binding by using HRP-conjugated sheep anti-dig antibodies.

Activation of the alternative pathway.

In order to enable the detection of all complement activation pathways in one assay system, the activation of the alternative pathway in an ELISA system was also studied. In contrast to the LP and the CP, activation of the AP is calcium-independent. Therefore, a calcium-free buffer was used, thus excluding involvement of the CP and the LP. As previously described (Fredrikson G.N., *et al.*, *J. Immunol. Methods* 166, 263-270, 1993), incubation of NHS in a buffer containing EGTA and Mg^{++} on plates coated with LPS resulted in a dose-dependent deposition of C3 (Fig. 4A). Some activation of C3 was also observed on plates coated with BSA only, most likely due to spontaneous activation of the AP. Surprisingly, a strong activation of C3 was also observed when NHS was incubated on mannan-coated plates by using the same conditions, suggesting that mannan may also support the activation of the AP (Fig. 4A). The detection of C3 was reduced until background levels when EDTA was present in the complement source (not shown). As expected from an AP-dependent mechanism, C3 activation in calcium-free buffers required a serum concentration that is about 10-fold higher than that required for C3 activation by mannan in a calcium-containing buffer *via* the LP (compare Fig. 4A with Fig. 3B). Although C3 activation was clearly detectable in a calcium-free buffer, no activation of C4 could be established (Fig. 4B), suggesting that under these conditions activation of C3 is independent of MBL binding and C4 activation.

EXAMPLE 5. Assessment of functional classical pathway activity by ELISA.

The protocol for the functional activity of the classical pathway was similar to the protocol for the LP assay, Example 3, with important modifications. As a ligand for CP activation, human IgM was coated at 2 µg/ml. After blocking of residual binding sites, serum samples, diluted in GVB++, were added to the plate and incubated for 1 hour at 37 °C. Complement binding was assessed by using dig-conjugated mAb directed against C1q, C4, C3, and C5b-9, followed by the detection of mAb binding by using HRP-conjugated sheep anti-dig antibodies.

CLAIMS

1. A method of functionally determining deficiencies in the complement system by only assaying the classical pathway, the alternative pathway, or the lectin pathway, respectively, the method comprising the steps of

(a) providing a sample of mammalian blood, serum, plasma, or another body fluid;

(b) selecting one pathway to be assayed;

(c) preventing in the sample the activation of the two non-assayed pathways;

(d) activating in the sample the pathway to be assayed; and

(e) determining in the sample any activation of the complement pathway.

2. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample an antibody against C1q of the complement system.

3. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a peptide inhibiting C1q of the complement system.

4. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a inhibitory C1q-binding protein.

5. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a small inhibitory C1q-binding molecule.

6. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample an antibody directed against C1r or C1s.

7. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a peptide inhibitor of C1r or C1s.

8. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a protease inhibitor of C1r or C1s.

9. The method as in claim 1, wherein under (c) the activation of the classical pathway is prevented by providing in the sample a potentiator of the C1 inhibitor.

10. The method as in claim 1, wherein under (c) the activation of the classical pathway and the lectin pathway is prevented by providing in the sample a Ca^{2+} binding agent.

11. The method as in claim 10, wherein the binding agent is a chelating agent.

12. The method as in claim 1, wherein under (c) the activation of the alternative pathway is prevented by dilution of the sample.

13. The method as in claim 1, wherein under (c) the activation of the alternative pathway is prevented by providing in the sample a protease inhibitor of factor D of the complement system.

14. The method as in claim 1, wherein under (c) the activation of the alternative pathway is prevented by providing in the sample an antibody directed against factor D of the complement system.

15. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample a carbohydrate or a derivative thereof.

16. The method as in claim 15, wherein the carbohydrate is mannose.

17. The method as in claim 15, wherein the carbohydrate derivative is a carbohydrate conjugate.

18. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample an antibody directed against MBL.

19. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample an antibody directed against a MASP protein.

20. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample a protease inhibitor of a MASP protein.

21. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample a peptide inhibitor of a MASP protein.

22. The method as in claim 1, wherein under (c) the activation of the lectin pathway is prevented by providing in the sample a peptide inhibitor of MBL.

23. The method as in claim 1, wherein under (d) the classical pathway is activated by providing in the sample at least one immunoglobulin.

24. The method as in claim 23, wherein the at least one immunoglobulin is immunoglobulin IgG or IgM.

25. The method as in claim 1, wherein under (d) the classical pathway is activated by providing in the sample an antibody directed against C1q.

26. The method as in claim 1, wherein under (d) the classical pathway is activated by providing in the sample a natural ligand of C1q.

27. The method as in claim 1, wherein under (d) the classical pathway is activated by providing in the sample a C1q-binding peptide.

28. The method as in claim 1, wherein under (d) the alternative pathway is activated by providing in the sample a lipopolysaccharide or a derivative thereof.

29. The method as in claim 1, wherein under (d) the alternative pathway is activated by providing in the sample a carbohydrate or a derivative thereof.

30. The method as in claim 29, wherein the carbohydrate is inulin.

31. The method as in claim 1, wherein under (d) the lectin pathway is activated by providing in the sample a mannan.

32. The method as in claim 1, wherein under (d) the lectin pathway is activated by providing in the sample a MBL-binding carbohydrate.

33. The method as in claim 32, wherein the MBL-binding carbohydrate is mannan.

34. The method as in claim 29 and 32, wherein the carbohydrate derivative is a synthetic carbohydrate conjugate.

35. The method as in claim 1, wherein under (d) the lectin pathway is activated by providing in the sample a ficolin-binding carbohydrate.

36. The method as in claim 1, wherein under (e) any activation of the complement pathway in the sample is determined by establishing an activitation of a complement protein from C4 to C9 of the same.

37 The method as in claim 36, wherein the activitation of a complement protein is established by providing in the sample antibodies against a formed C5b-9 complex.

ABSTRACT

In a method of functionally determining deficiencies in the complement system by only assaying the classical pathway, the alternative pathway, or the lectin pathway, respectively, the method comprising the steps of (a) providing a sample of mammalian blood, serum, plasma, or another body fluid; (b) selecting one pathway to be assayed; (c) preventing in the sample the activation of the two non-assayed pathways; (d) activating in the sample the pathway to be assayed; and (e) determining in the sample any activation of the complement pathway.

Fig 1.

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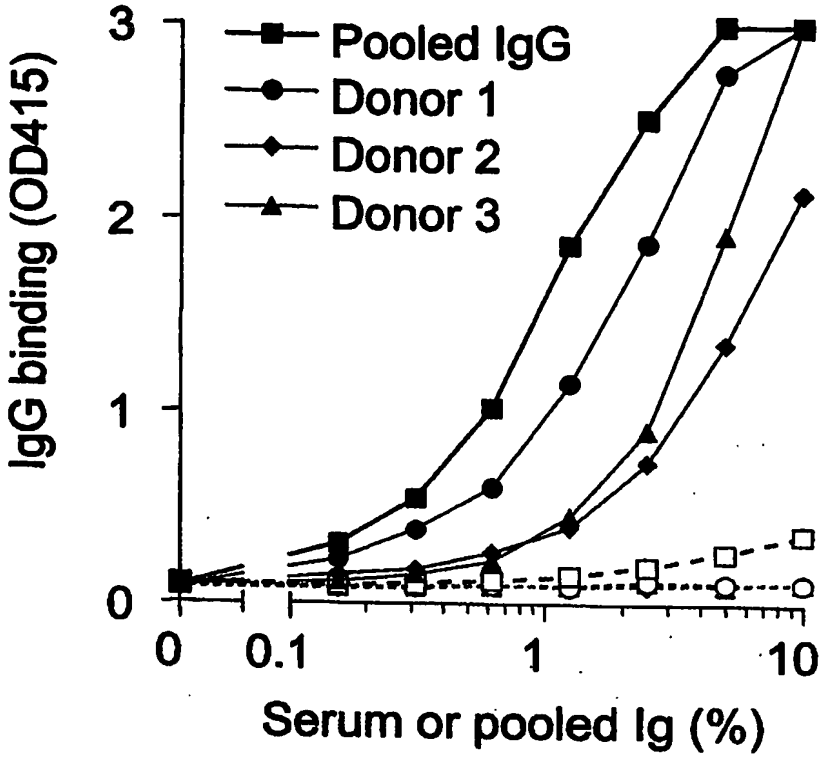

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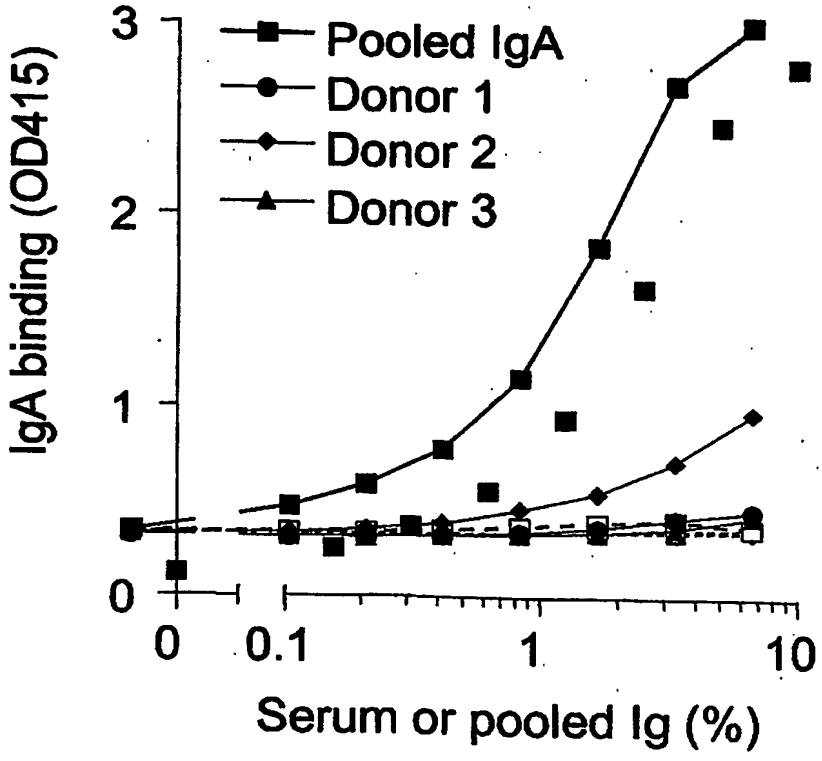

Fig 1.

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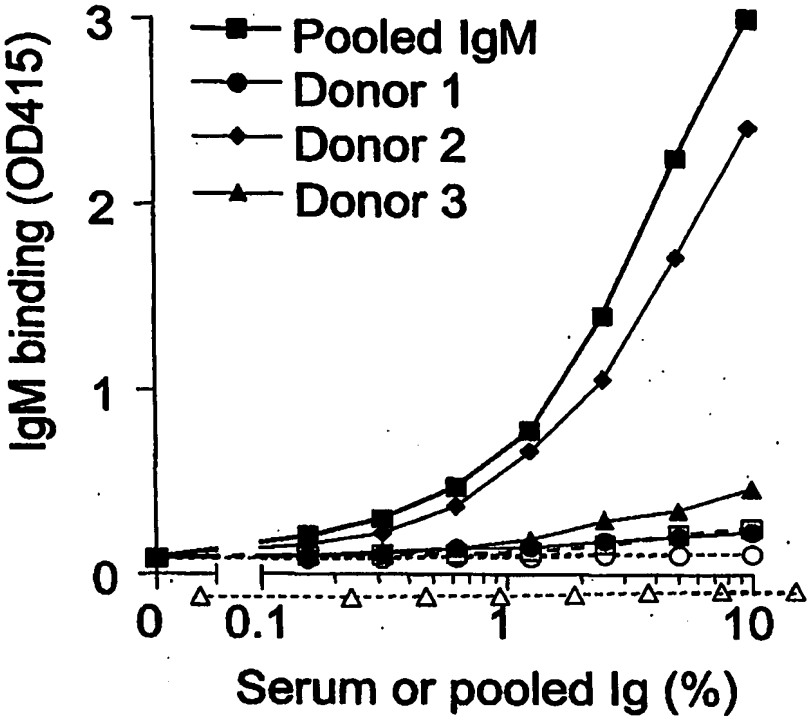

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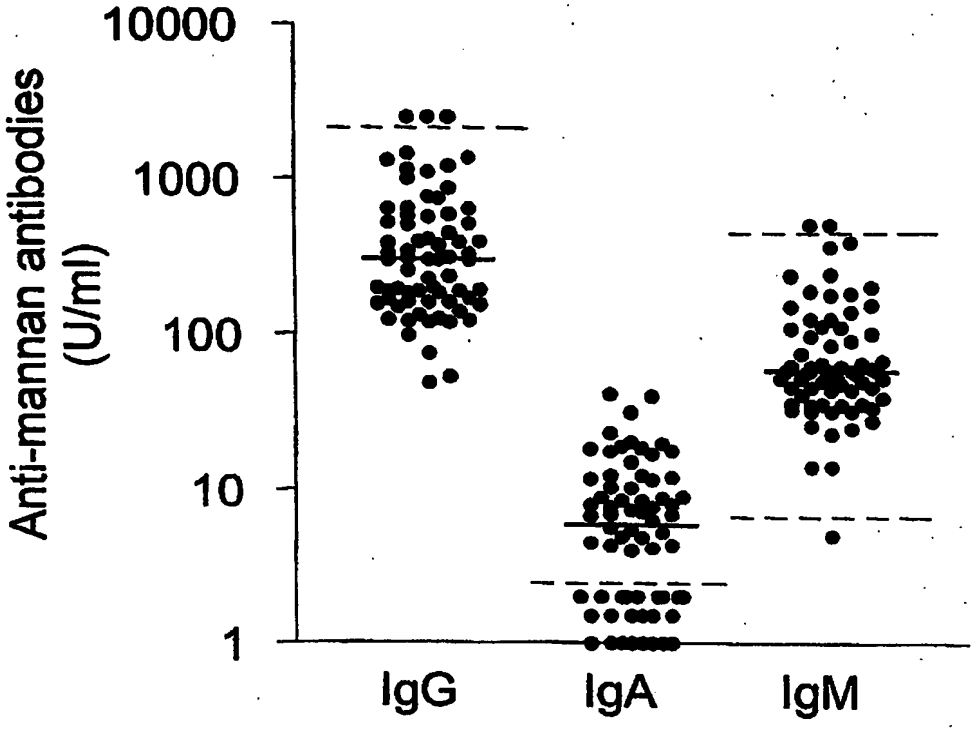

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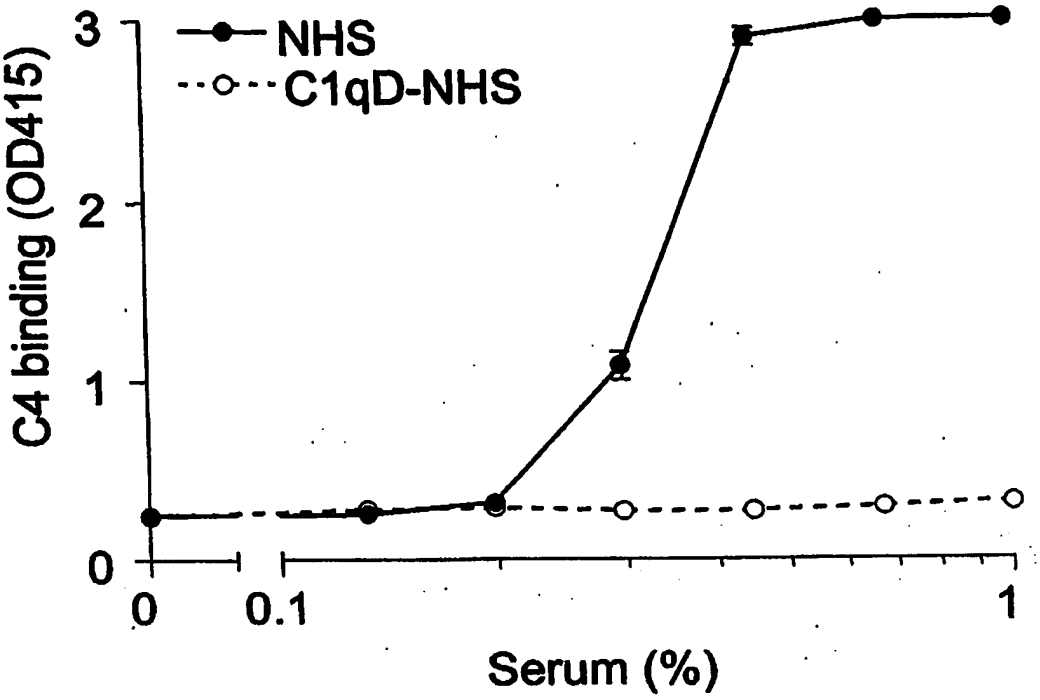

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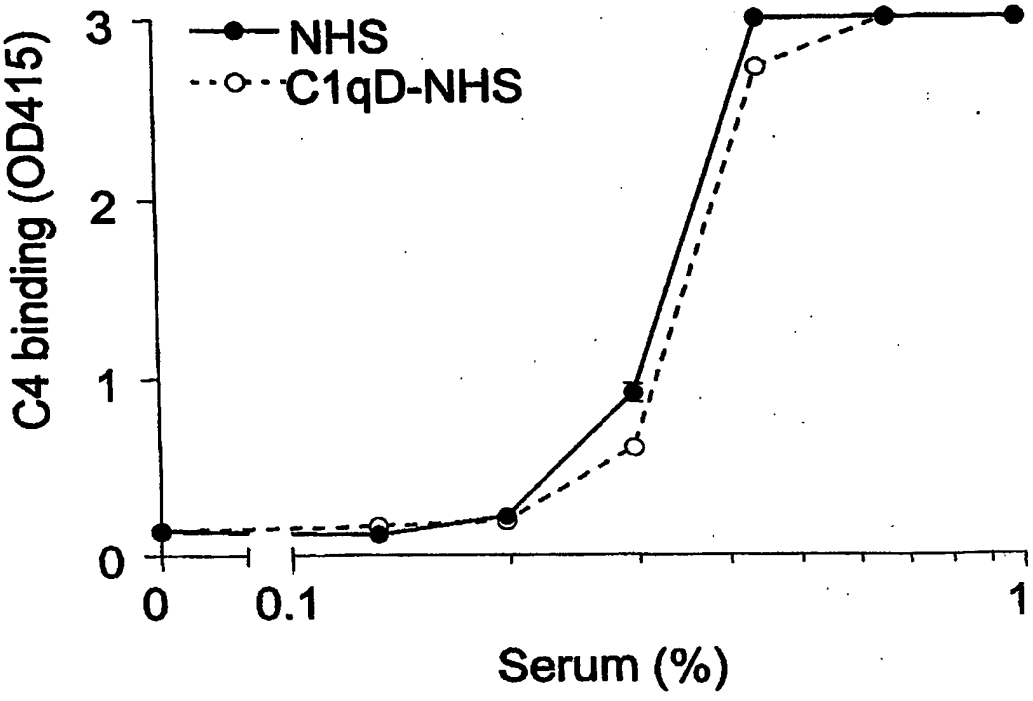
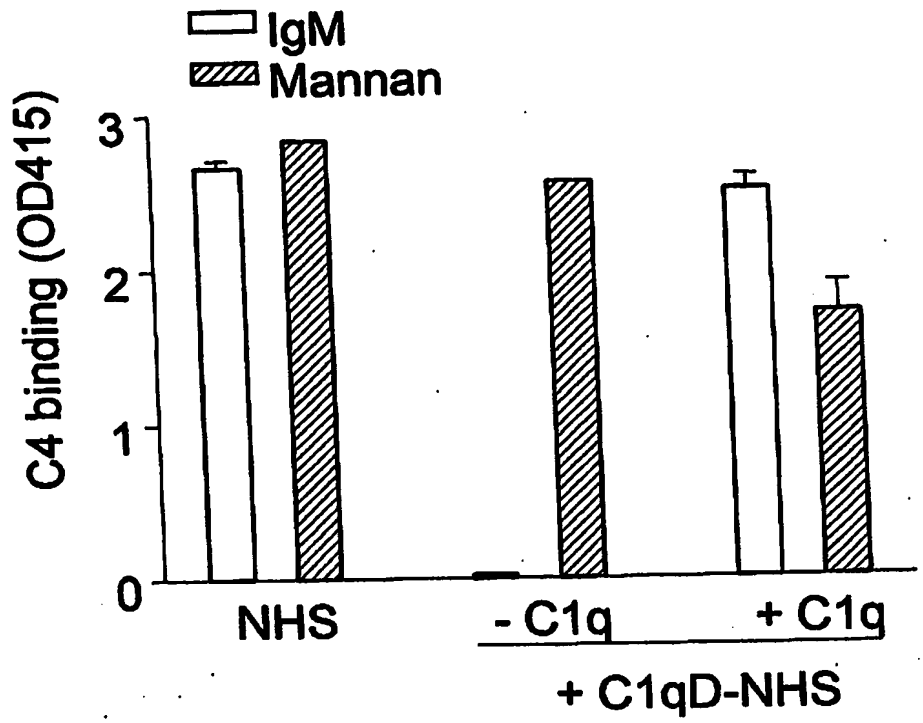


Fig 2.

C



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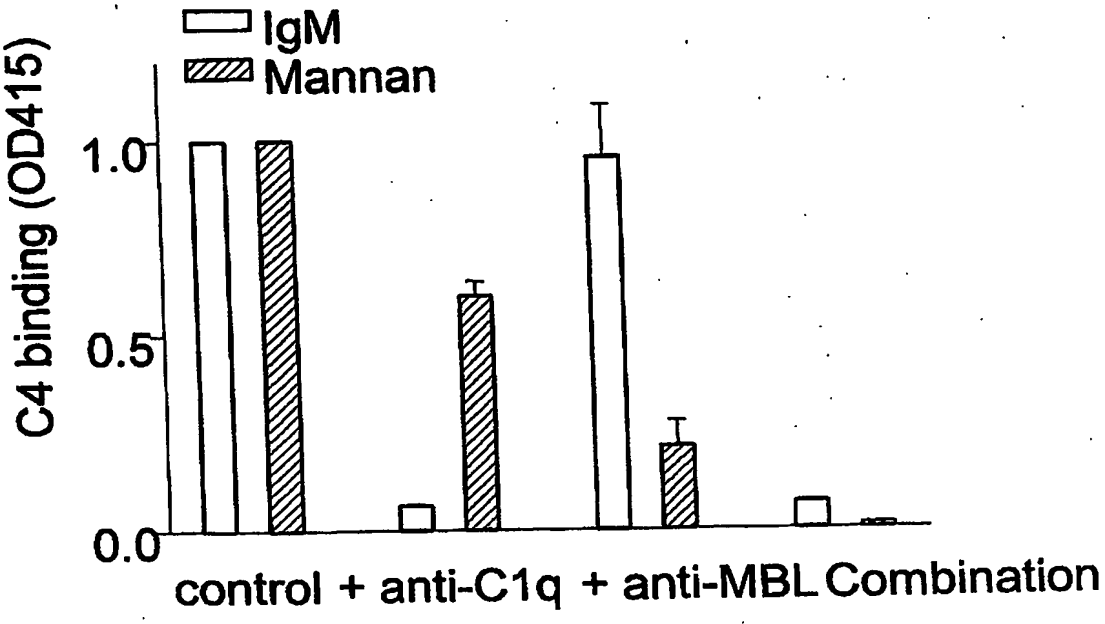

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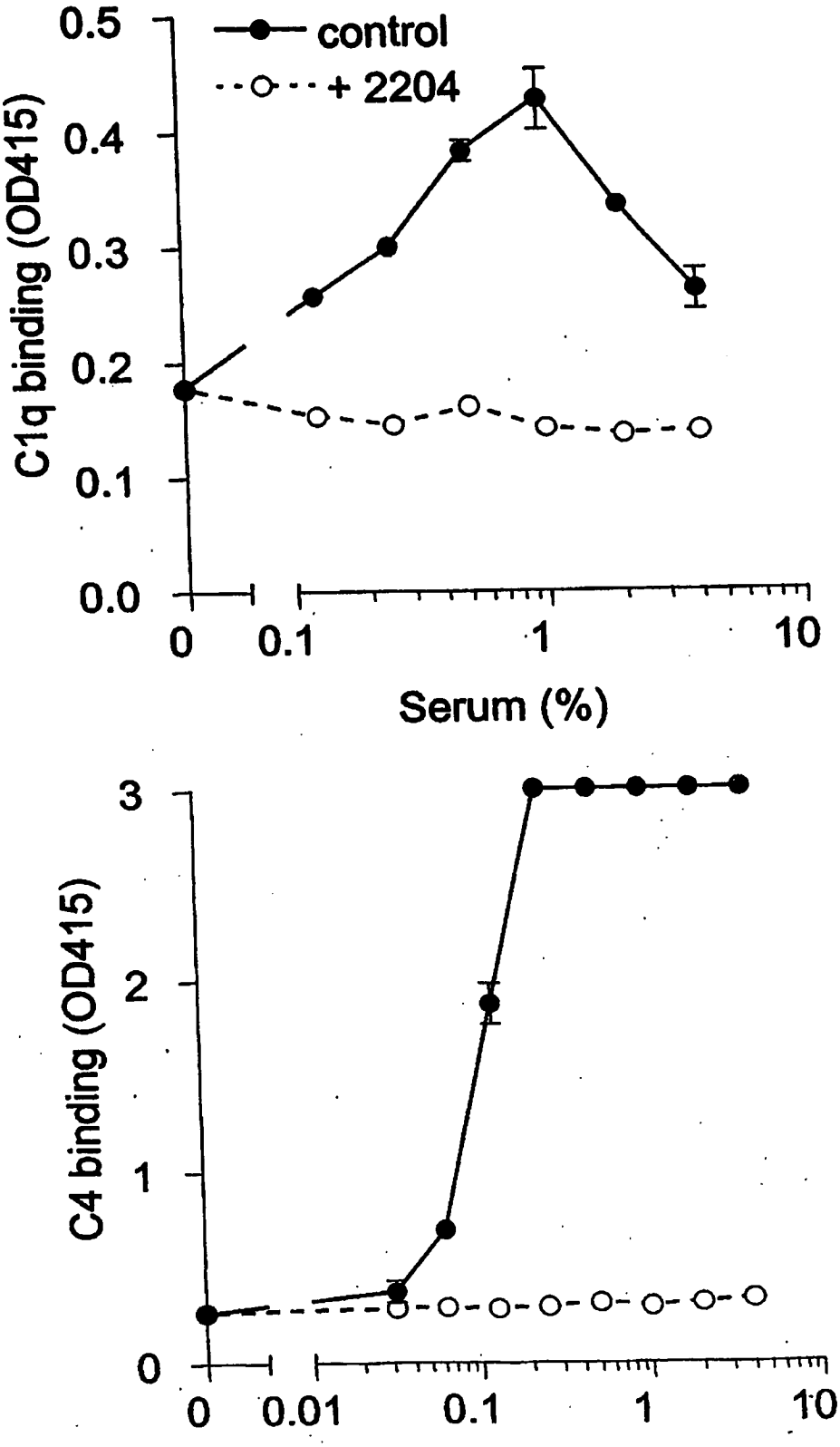
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C1q binding (OD415)
0.5
0.4
0.3
0.2
0.1
0.0
0
0.1
1
10
Serum (%)
C4 binding (OD415)
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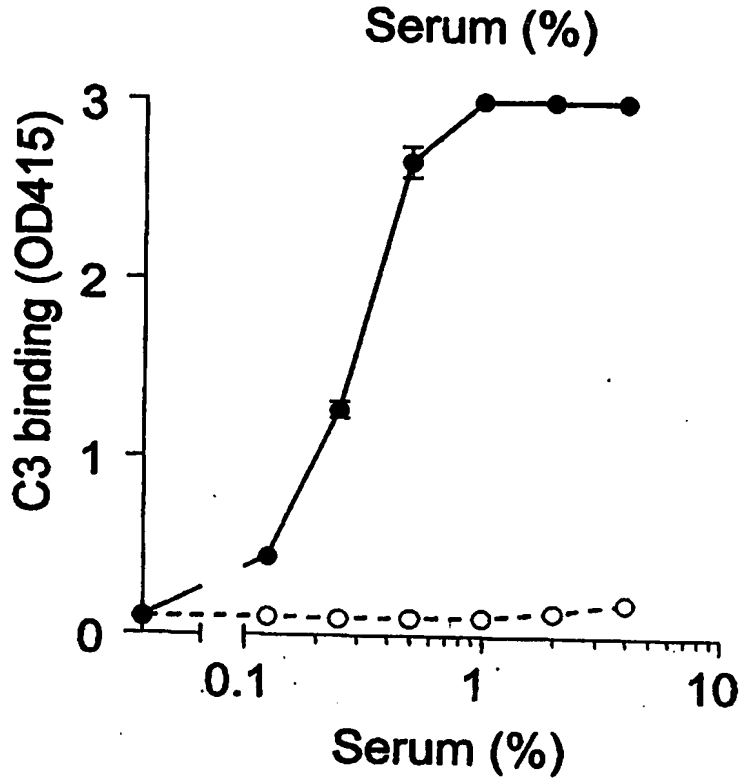
2
1
0
0
0.01
0.1
1
10

Fig 3.

A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2
1
0
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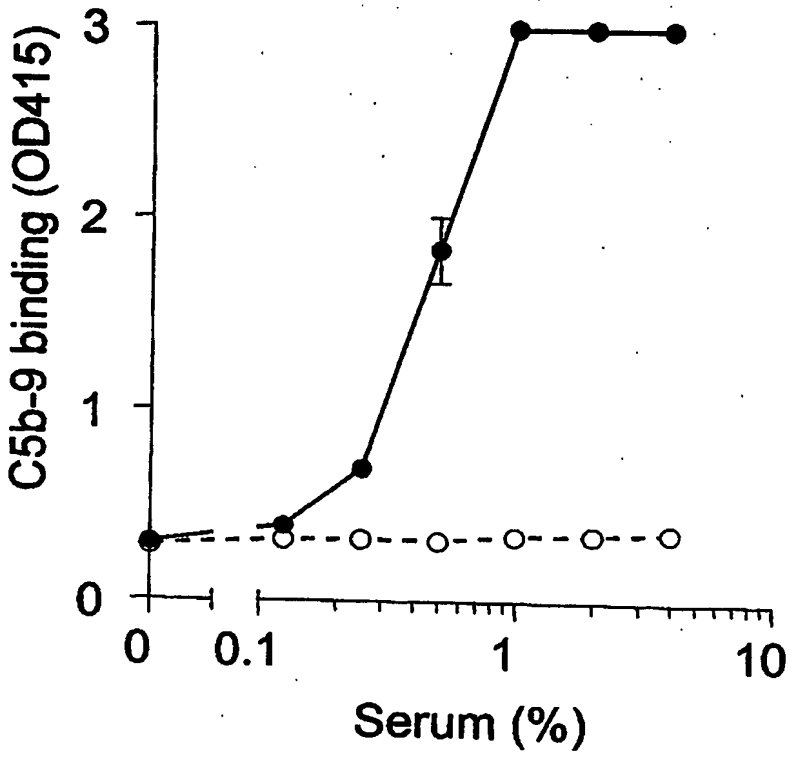
1
10
Serum (%)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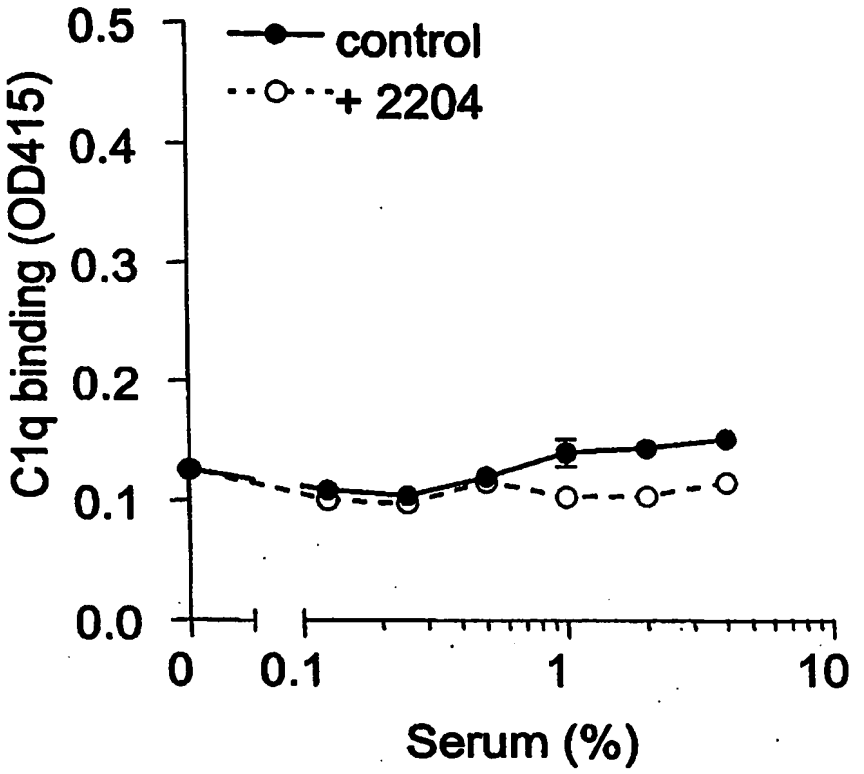
1
10
Serum (%)

Fig 3.

B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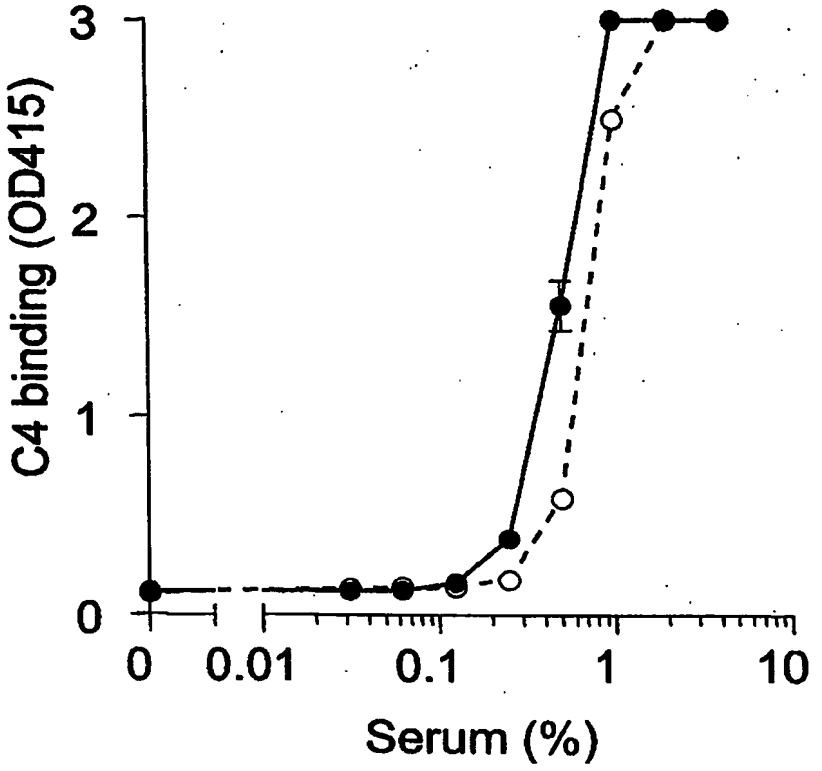
0
0.1
1
10
Serum (%)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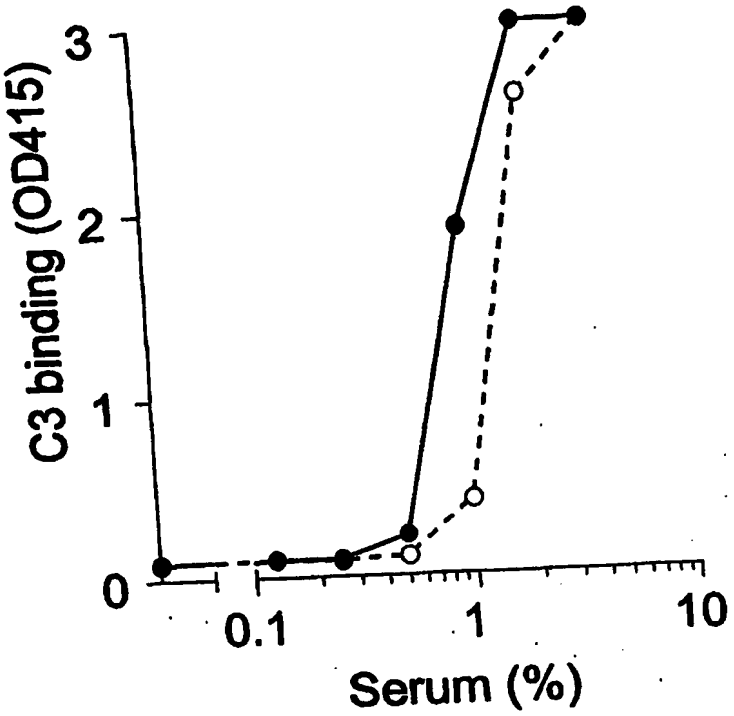
1
10
Serum (%)

Fig 3.

B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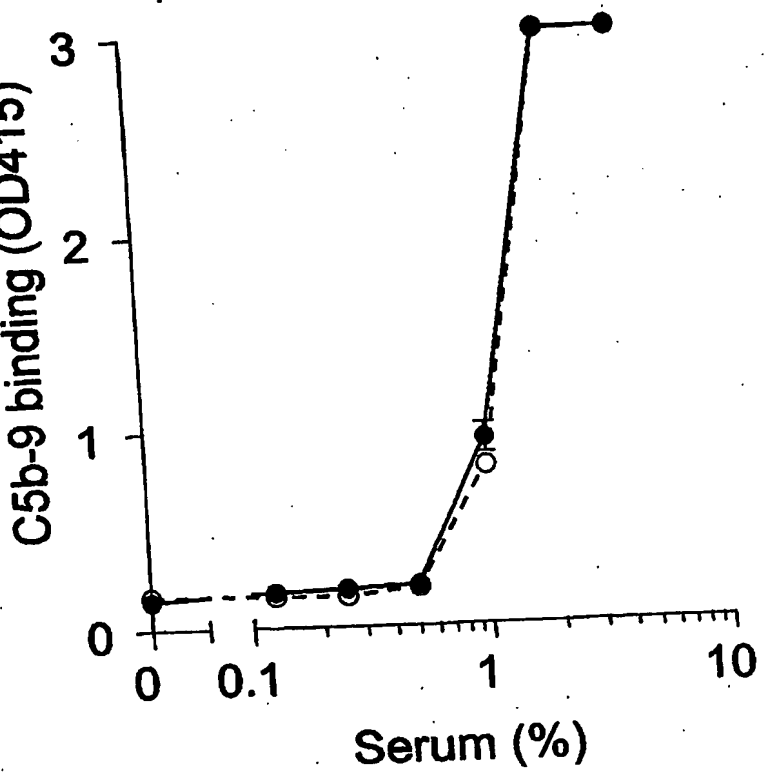
10
Serum (%)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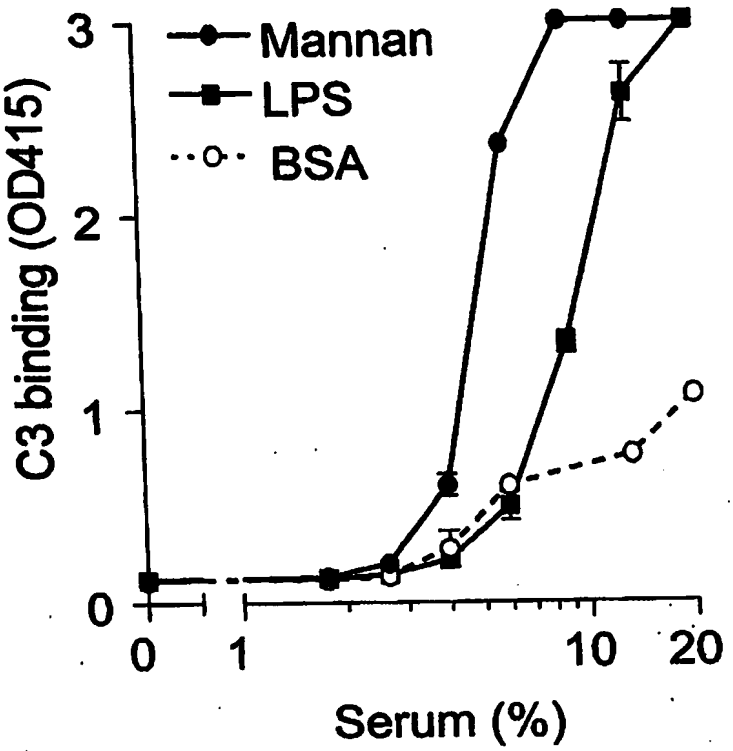
10
Serum (%)

Fig 4.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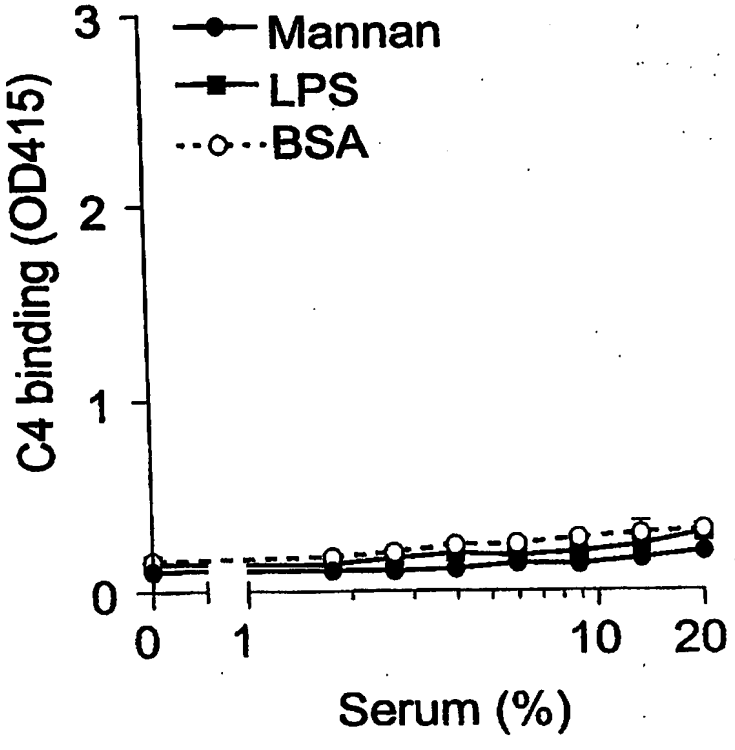
1
10
20
Serum (%)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C4 binding (OD415)
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1
10
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Serum (%)

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